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Biolistic inoculation of gladiolus with cucumber mosaic cucumovirus

Joan A. Aebig[1], Kathryn Kamo, Hei-Ti Hsu*

Floral and Nursery Plants Research Unit, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705-2350, USA

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Abstract

A new method of inoculation of gladiolus with cucumber mosaic virus (CMV) was developed using the Bio-Rad Helios Gene Gun System. This method circumvents the traditional use of aphids to transmit CMV, a virus that is mechanically transmissible to many plant species but only with difficulty to gladiolus. Cartridges containing virus-coated gold microcarriers were prepared and the virus shot into *Nicotiana benthamiana* leaves and gladiolus corms and cormels. The biolistic procedure successfully transmitted three CMV isolates, two from serogroup I and one from serogroup II. Survival rates of two cultivars of gladiolus cormels and corms in sterile and non-sterile environments were compared. Infection rates of 100% were obtained when as little as 2 μg of virus was used in cartridge preparation. CMV remained viable after the cartridges were stored for many months at 4 °C.

Keywords: Particle bombardment; Plant virus inoculation; Virus-coated gold particles

1. Introduction

Gladiolus is an important ornamental crop in the U.S. and worldwide. A major pathogen of gladiolus is cucumber mosaic virus (CMV), a broad host range cucumovirus, which infects more than 1000 species of plants (Chen, 2003). More than 60 species of aphids, especially *Aphis gossypii* and *Myzus persicae*, naturally spread CMV (Kennedy et al., 1962). The mode of aphid transmission is non-persistent, or stylet-borne (Kaper and Waterworth, 1981). The V-strain of CMV, however, was found to be transmitted by *Myzus* sp. in a semi-persistent manner to *Phaseolus vulgaris* (Kameya-Iwaki et al., 2001). There are many reports of the seed transmission of CMV but not in gladiolus (Kaper and Waterworth, 1981). The virus is also mechanically transmitted to many plant species but generally not to gladiolus. Leaves of infected gladiolus are usually symptomless while symptoms on flower petals vary from a few streaks to severe color break and deformation (Stein, 1995).

For many crops, weeds are important in the epidemiology of CMV disease. Aphids acquire the virus while feeding on infected weeds surrounding crop fields, and the seeds of infected weeds harbor the virus over the winter (Kaper and Waterworth, 1981). However, this may not be the case with gladiolus. One study from Israel reports that aphids transmit CMV from gladiolus to gladiolus, not from four other source plants tested (Aly et al., 1986). Thus, the aphid transmission of CMV is extremely plant-host specific. Furthermore, in fields of gladiolus, CMV is spread by several species of aphids with different cultivars of *Gladiolus grandiflorus* exhibiting varying degrees of susceptibility (Aly et al., 1986). The source, then, of CMV infection in fields of gladiolus is previously infected gladiolus, in which corms maintain the virus during dormancy and produce infected leaves each season to feeding aphids.

In studies of CMV infection in gladiolus and disease resistance, transmission is performed by aphid feeding and inoculation. Performing aphid transmission studies is laborious and includes maintenance of a virus-free aphid colony and

* Corresponding author. Tel.: +1 301 504 5657; fax: +1 301 504 5096.
E-mail address: hsuht@ba.ars.usda.gov (H.-T. Hsu).
[1] Present address: NIH/NIAID/MVDU, 5640 Fishers Lane, Rockville, MD, USA.

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propagation of an isolate of vectorial CMV, which must always be aphid-transmissible. Furthermore, there exists the danger that viruliferous aphids might escape from containment cages. It would be extremely useful to have a convenient method of inoculating gladiolus without the use of aphids.

Hoffmann et al. (2001) introduced a new method for the inoculation of potato leafroll polerovirus, a phloem-limited, aphid-borne virus, previously thought to be non-mechanically transmissible. The Bio-Rad Helios Gene Gun System (Bio-Rad, Hercules, CA, USA) was used to shoot virus-labeled gold particles into two *Nicotiana* spp. resulting in systemic infection, although infection in *Physalis* was not achieved.

Successful biolistic inoculation (particle bombardment) of virus particles has also been demonstrated for barley yellow dwarf luteovirus in wheat (Helleco-Kervarrec et al., 2002). Inoculation of grapevine fanleaf nepovirus using the technique was attempted, but infection was not established in *Vitis* sp. (Valat et al., 2003).

In the present study, a procedure is developed for the biolistic inoculation of CMV into gladiolus cormels and corms.

2. Materials and methods

2.1. Reagents and viruses

Unless otherwise noted, all reagents were from Sigma, St. Louis, MO, USA.

All CMV cultures used in the study were obtained under a permit issued by USDA-APHIS. CMV-Fny was from Milton Zaitlin of Cornell University, Ithaca, NY, USA and CMV-S was from Jacobus M. Kaper of the Agricultural Research Service at Beltsville, MD, USA.

CMV-glad was isolated at Beltsville from Peter Pears gladiolus obtained from Gary Wilfret of Manatee Fruit, Bradenton, FL, USA. It was identified using the ImmunoStrip Test for CMV (Agdia Inc., Elkhart, IN, USA) but was not further characterized. Infected leaves were ground in 2.5 mM sodium borate buffer containing 2.5 mM EDTA, 10 mM sodium phosphate, and 0.2% sodium sulfite pH 8. Celite was added to the inoculum, and the virus was mechanically inoculated onto $1\frac{1}{2}$-month-old *Nicotiana benthamiana* leaves. Four weeks later, *N. benthamiana* leaves showing mosaic symptoms were used to inoculate additional *N. benthamiana* plants, and nine days later the virus was purified.

2.2. Virus purification and storage

CMV isolates were purified according to the method of Roossinck and White (1998). Briefly, leaf tissue was ground in a blender with extraction buffer (0.5 M sodium citrate, pH 7.0, 5 mM EDTA, 0.5% thioglycolic acid) and chloroform. The aqueous phase was collected and filtered. The virus was further purified by ultracentrifugation through two successive 10% sucrose cushions. Optical density was determined by spectrophotometry and the concentration determined assuming an extinction coefficient of 5.

Purified virus was stored in 50% glycerol at −20 °C.

2.3. Gene gun and preparation of cartridges

The Helios Gene Gun System (Bio-Rad, Hercules, CA, USA) was used according to manufacturer's instructions.

2.3.1. Preparation of cartridges without the use of spermidine

Cartridges containing virus-coated gold microcarriers were prepared according to manufacturer's procedure with modification. Briefly, 100 μg or less of purified virus in 50% glycerol was diluted in 5 mM borate buffer, pH 9 to bring the glycerol concentration to less than 18% and the volume to 100 μl. (If the glycerol concentration was too high, the virus solution was either dialyzed against 5 mM borate buffer, pH 9 or repeatedly filtered and diluted in a pre-rinsed YM-100 Centricon filter (Millipore Corp., Bedford, MA, USA)). The 100 μl volume was added to 15 mg gold microcarriers (0.6 μm size, Bio-Rad) in a 1.5 ml microfuge tube. No spermidine was used. The mixture was sonicated for 5 s. Fifty microliters of 1 M $CaCl_2$ was added drop-wise while vortexing. This mixture was incubated for 10 min at room temperature. The virus-coated gold particles were pelleted in a microfuge at top speed for 15 s, washed three times in 1 ml absolute ethanol (newly opened bottle, Warner–Graham Company, Cockeysville, MD, USA), and finally resuspended in 3.5 ml ethanol containing 0.1 mg/ml PVP (MW 360,000, Bio-Rad).

Within 1 h, the virus-coated microcarriers were coated onto gold-coat tubing (Bio-Rad) according to the manufacturer's procedure. The microcarriers never coated the entire inner surface of the tubing, but rather deposited as an even trough along the bottom of the tubing. One and a half inch cartridges were cut from the tubing and stored at 4 °C in a vial with desiccant for up to 15 months. For sterile cartridges, the 1/2-in. tubing sections were cut with a sterile razor blade in a biological safety cabinet.

2.3.2. Preparation of cartridges using spermidine

In some experiments, spermidine, which is used in the precipitation of DNA into gold, was included in the gold-coating procedure. Fifty microliters of purified virus was added to 50 μl 0.05 M spermidine. This 100 μl volume was added to 15 mg gold microcarriers, and the procedure continued as above.

2.4. Preparation of plants for biolistic inoculation

2.4.1. Gladiolus grandiflora cvs.

Peter Pears and Jenny Lee were obtained from Manatee Fruit and Oglesby Plant Laboratory, Altha, FL, USA, respectively. Gladiolus cormels and corms, generally 10–25 mm in diameter, were germinated either in well-drained soil or

vermiculite in pots in the greenhouse or in the lab under fluorescent light. Sterile gladiolus cormels, 5–10 mm in diameter, were germinated in Murashige and Skoog's medium with vitamins (Murashige and Skoog, 1962; M5519, Sigma Co., St. Louis, MO, USA or PhytoTechnology Laboratories, Shawnee Mission, KS, USA) containing 3% sucrose and 0.2% Phytagel, pH 5.8, in Magenta jars. The jars were placed on shelves with a 16-h light photoperiod from fluorescent lights (15 μmol m^{-2} s^{-1}) in a tissue culture room.

For both sterile and non-sterile gladiolus with actively growing leaves, the leaves and brown coat were removed with a scalpel or razor blade. Care was taken not to damage the meristem at the top of the cormel. Between 0 and 2 mm of leaves were left on top of the cormel. In some experiments, the cormels with leaves and brown coat removed were left uncovered in a sterile Petri plate placed in a biological safety cabinet to dry for 30–45 min.

All gladioli used for CMV inoculation were first tested for the absence of CMV infection either in a prior growing season, or when the leaves were excised immediately prior to inoculation or both. Leaves were tested for CMV infection using the ImmunoStrip Test for CMV (Agdia Inc., Elkhart, IN, USA). All of the gladioli were confirmed negative for CMV prior to inoculation.

Suitable containers for supporting the cormels were devised. For non-sterile cormels, corkborers of various sizes were used to cut holes in a styrofoam tray covered with parafilm. Any roots were tucked under the bulb so as not to be damaged by the burst of helium from the gene gun. A 96-well plate was autoclaved for 5 min to deform the polystyrene and produce wells of various oval shapes and sizes to support the sterile cormels. A well with a diameter slightly smaller than that of the cormel was used so that the cormel would not become lodged in the bottom of the well following the shot. Whatman 3M filter papers with holes punched in them were sterilized by autoclaving and used to cover the 96-well plate, exposing only the top of the cormel to the gene gun shot. The filter paper was optional, but it helped protect the roots and absorbed the virus-coated gold particles that landed outside the cormel. For sterilization, the gene gun was wiped with ethanol in a biological safety cabinet. The cartridge holder, barrel liner and cartridge extractor tool were sterilized by autoclaving for 5 min.

In preparation for shooting, *N. benthamiana* leaves were supported by placing a parafilm-coated box under the leaf and pressing gently on the leaf with the gun's spacer to flatten the leaf against the parafilm, taking care not to indent or crush the leaf.

2.5. Gene gun inoculation

The barrel liner provided with the Helios system determined the distance from the gene gun to the cormel. This distance was 2.5 cm. Firing pressure was adjusted to 380–480 psi for the gladiolus with one cartridge being shot down into the top of each cormel in the meristem region. As a positive control for virus viability, one cartridge was shot at 100–110 psi into one leaf of a 2-month-old *N. benthamiana* plant.

2.6. Diagnosis of CMV infection

At least 3 weeks after inoculation, newly emergent gladiolus leaves were tested for CMV using the ImmunoStrip Test following the manufacturer's procedure.

N. benthamiana plants were observed for symptom development during the 2-week post-inoculation period. Mosaic symptoms indicated CMV infection. Milder infections, which resulted in only leaf curl were confirmed positive by the ImmunoStrip Test.

3. Results

3.1. Cartridge preparation procedure

Initial experiments using 30 mg of gold microcarriers in cartridge preparation and a firing pressure of only 100 psi caused severe damage of *N. benthamiana* leaves. The entire 1/2-in. diameter target area became necrotic from the damage of the shot, resulting in no virus infection. In subsequent experiments, 15 mg of gold microcarriers was used, and the virus and $CaCl_2$ volumes were adjusted accordingly. Final resuspension in 3.5 ml ethanol filled the length of tubing but reduced the amount of microcarriers in each cartridge, resulting in less leaf damage.

3.2. Virus quantity in cartridges

In a preliminary experiment to determine the amount of virus that was actually deposited in a cartridge, spent solutions from the microcarrier and cartridge preparation procedures were saved. In this experiment, in which 100 μg of CMV-Fny was precipitated into 11 mg microcarriers, the 0.15 ml leftover solution containing $CaCl_2$ was saved and, within 45 min, diluted in 5 mM borate buffer, pH 8 and mechanically inoculated into two *N. benthamiana* for each dilution. In 2 weeks, symptoms developed on plants inoculated with the 1:100 or 1:1000 dilutions (data not presented). Symptoms were also observed on one of the plants inoculated with the 1:10,000 dilution of the saved $CaCl_2$ solution. The results indicate that not all the virus precipitated into the microcarriers. Plants inoculated with the 1:100,000 dilutions did not develop symptoms. When the experiment was repeated using 155 μg of virus, one-third of the *N. benthamiana* plants inoculated with the 1:10,000 dilution of the saved $CaCl_2$ solution became infected. For this preparation of purified CMV-Fny, infection in *N. benthamiana* occurs in one-fourth of the hand-inoculated plants when 0.05 μg/ml is used. None are infected when 0.008 μg/ml is used as inoculum. Back calculation then estimates the amount of virus left over in the $CaCl_2$ solution to be 0.05 μg/ml $\times$ 10,000 $\times$ 0.15 ml = 75 μg. Similarly, from the same experiment, the leftover ethanol so-

Table 1
Effect of spermidine on the viability of CMV-glad isolate in cartridge preparations as determined by biolistic inoculation into *Nicotiana benthamiana*

Amount of virus in preparations (μg)	Spermidine	Number of plants positive for CMV infection/number of plants inoculated
4	+	0/1
20	+	0/1
100	+	0/1
0.4	–	0/4
2	–	4/4
10	–	4/4
50	–	4/4

lution containing CMV-labeled microcarriers following the tubing coating procedure was saved. Leftover microcarriers were pelleted in a microfuge for 10 s, the ethanol discarded, and the pellet air-dried for 10 min. The microcarriers were resuspended in 0.6 ml borate buffer and inoculated by hand into two *N. benthamiana* plants. Within 2 weeks, both plants developed viral symptoms. Thus, in this experiment, less than 25 μg of virus (out of 100 μg starting material) ended up in the tubing, with no more than 0.62 μg per cartridge. Twelve *N. benthamiana* plants were shot with these cartridges, and 100% became infected.

In subsequent experiments, 15 mg microcarriers were used instead of 11 mg to improve virus–gold precipitation. Quantifying the amount of virus in each cartridge was discontinued as it proved to be difficult, probably variable, and therefore, very inaccurate. Also, frequently the virus–gold mixture did not coat evenly along the tubing, presumably due to water in the ethanol. In these cases, the tubing was discarded and the gold-coating procedure repeated. In general, the cartridge preparation procedure was highly variable, and only cartridges that had an even trough of gold deposited along their full 1/2-in. length were used in the gladiolus inoculation experiments.

In this report, virus quantities are not given per cartridge, but rather, the total amount of virus used in that experiment's cartridge preparation procedure is reported. One cartridge preparation procedure usually produced between 30 and 40 cartridges.

3.3. Effect of spermidine on CMV viability

In initial experiments, spermidine was included in the microcarrier coating procedure, and CMV-Fny was found to be viable when as little as 1 μg of virus was used in the preparation (results not shown). However, for the CMV-glad isolate, the inclusion of spermidine resulted in loss of viability in all preparations (Table 1) so was not used in subsequent experiments.

3.4. Survival rates of gladiolus

Because gladiolus bulbs are subject to rotting when damaged or over-watered, the survival rates of bulbs grown under various conditions following inoculation were determined. Plants grown in non-sterile soil in the greenhouse following inoculation survived long enough to produce a leaf for testing, but all the corms later rotted. Plants grown in non-sterile vermiculite on a lab bench where watering was better controlled had survival rates between 42 and 83%. The best survival rates were obtained for cormels grown and inoculated under sterile conditions (Table 2). The loss of sterile, inoculated cormels was attributed to damage caused by either the peeling of the brown coat, the excising of the leaves close to the meristem, or the shot itself. Between 89 and 94% of the sterile cormels survived the entire procedure. Although removal of the leaf close to the meristem is potentially damaging, it could not be avoided. The plants only became infected when the meristem region was the target of the Helios Gene Gun, not the leaf (results not shown).

Drying the cormels did not increase the rate of infection. For dry Peter Pears, 4/6 were infected and survival was 100%, and for non-dried Peter Pears, 5/5 were infected and 83% survived. For dry Jenny Lee, 4/4 were infected and 67% survived, and for non-dried Jenny Lee, 4/4 were positive and survival was 67%.

3.5. Minimum virus quantity to establish infection in N. benthamiana and gladiolus

The minimum quantity of CMV-glad that was used in the cartridge preparation procedure and assured 100% infection upon shooting into *N. benthamiana* was 2 μg (Table 1).

The minimum quantity of CMV-glad that was used in the cartridge preparation procedure and assured 100% infection in Peter Pears gladiolus was 2 μg (Table 3). In Jenny Lee glads, with the 2 μg preparation, 75% of cormels became infected, and 100% infection was achieved with the 10 μg preparation.

3.6. Alternate inoculation procedures

Three other methods of mechanically inoculating cormels with CMV were attempted. A 50 μg/ml solution of CMV-glad containing sterile celite was applied to the base of Peter Pear and Jenny Lee glad cormels using gloved fingers and rubbed on both sides of the leaves from the base to the tip in one stroke. All of the cormels survived the procedure and remained sterile. Two out of five (40%) of the Peter Pear glads and 2/7 (29%) of the Jenny Lee glads became infected (Table 4). Although encouraging, this procedure did not result in a high enough infection rate to be useful as a reliable inoculation method.

A similar procedure was tried using forceps instead of fingers to apply a 50 μg/ml solution of CMV-glad containing sterile carborundum and scraped along the front and back of the leaves. When a very light pressure was used, all the plants survived, but none became infected (Table 4). Applying more pressure caused severe mechanical injury and plant death.

Table 2
Survival of gladiolus cormels grown under various conditions following biolistic inoculation[a]

CMV isolate	Glad variety	Planting medium, location	Number of cormels inoculated	Number of plants surviving through testing	Survival rate[b] (%)
Fny	Peter Pears	Soil, greenhouse	4	0	0
Fny	Peter Pears	Soil, greenhouse	5	0	0
Glad	Jenny Lee	Vermiculite, lab	12	5	42
Glad	Peter Pears	Vermiculite, lab	12	7	58
Glad	Peter Pears	Vermiculite, lab	12	7	58
Glad	Jenny Lee	Vermiculite, lab	12	10	83
Fny	Peter Pears	MS media, sterile jars	17	16	94
Fny	Jenny Lee	MS media, sterile jars	9	8	89

None contained spermidine. Survival rate = percentage of plants that survived inoculation, testing, and subsequent growth.
[a] Between 10 and 100 μg of purified CMV-Fny or CMV-glad isolate was used in each cartridge preparation.
[b] Two and a half to nine weeks following inoculation, a small amount of newly emergent leaves was removed for testing, and the plants continued under their growth conditions and monitored for bulb survival.

Table 3
Infection rates of gladiolus corms inoculated with CMV-glad using the Helios Gene Gun System

Amount of virus in preparations[a] (μg)	Glad variety (12 of each)	Number of plants tested[b]	Infection rate[c] (%)
50	Peter Pears	7	100
	Jenny Lee	10	100
10	Peter Pears	7	100
	Jenny Lee	4	100
2	Peter Pears	3	100
	Jenny Lee	4	75
0.4	Peter Pears	9	11
	Jenny Lee	12	0

[a] Cartridges were prepared with 10, 5, 2, or 0.4 μg CMV-glad. Twelve cormels of each glad variety were inoculated with each preparation. Following inoculation, cormels were planted in vermiculite and grown on a lab bench.
[b] Number of plants which survived long enough to put out a new leaf for testing.
[c] Infection rate is the percentage of plants testing positive for CMV per the number of plants tested.

A final method of inoculation was attempted using insect pin pricking on gladiolus leaf or meristem through a drop of CMV-glad solution at 50 μg/ml. Survival rates varied, and none of the plants became infected. An example of one experiment is shown in Table 4.

3.7. Viability of cartridges following storage

Cartridges made with CMV-Fny (with or without spermidine) were stored in vials with desiccant at 4 °C for $8\frac{1}{2}$–$15\frac{1}{2}$ months, then, shot into *N. benthamiana* plants, one cartridge per plant. All plants became infected within 7 days following inoculation (Table 5).

The progression of symptoms in *N. benthamiana* resulting from CMV infection began with excessive curling of the uppermost leaves followed by the appearance of mild mosaic progressing into a more severe mosaic. Plants shot with cartridges prepared from larger amounts of CMV-Fny (100 or 150 μg) developed mosaic symptoms sooner than those shot with cartridges prepared from lesser amounts of virus (Table 5). Although determining the exact amount of virus in a cartridge is difficult as previously discussed, the expected

Table 4
Comparison of three mechanical inoculation methods with the Helios Gene Gun inoculation method of CMV-glad isolate

Inoculation method[a]	Glad variety	Number of plants inoculated	Number of plants infected	Survival rate (%)
Finger (and celite)	Peter Pears	5	2	100
	Jenny Lee	7	2	100
Forceps (and carborundum)	Peter Pears	7	0	100
	Jenny Lee	6	0	100
Pin	Peter Pears	6	0	100
	Jenny Lee	6	0	83
Biolistic Helios Gene Gun	Peter Pears	6	5	83
	Jenny Lee	6	4	67

[a] For the finger, forceps and pin pricking inoculations, the virus was at a concentration of 50 μg/ml. Inoculation using the Gene Gun was with a 10 μg preparation of cartridges. All cormels were grown and inoculated under sterile conditions.

Table 5
Viability of CMV-Fny cartridges. Cartridges stored at 4 °C were shot into *N. benthamiana*, and the symptoms were recorded at 5, 7, and 8 days post-inoculation (dpi)

Amount of virus used in cartridge preparation (μg)	Length of storage (months)	Symptoms		
		5 dpi	7 dpi	8 dpi
20	$15\frac{1}{2}$	Mild mosaic	Mosaic	Mosaic
100	14	Mild mosaic	Mosaic	Mosaic
1	14	None	Leaf curl	Mild mosaic
100	9	Mosaic	Mosaic	Mosaic
150	9	Mosaic	Mosaic	Mosaic
1	$8\frac{1}{2}$	None	Mild mosaic	Mosaic

trend in symptom development based on dose of virus given is evident.

3.8. Biolistic inoculation of CMV-S into gladiolus

CMV-S is a serogroup II strain of CMV. As there have been no reports of a serogroup II strain of CMV isolated from gladiolus, it was of interest to determine if CMV-S would infect gladiolus. Cartridges prepared with 100 μg of purified CMV-S were shot into 25 Peter Pears cormels in tissue culture. After 3 weeks, 22 of the 25 cormels put out leaves large enough to be tested. Twenty were positive for CMV and two were negative. CMV-S was also shot into non-sterile Jenny Lee cormels and established an infection.

4. Discussion

CMV is among the many plant viruses readily transmitted through wounds on plant surfaces by mechanical inoculation. Certain abrasives added to the inoculum or dusted on the surface of the leaves before inoculation greatly improve the efficiency of mechanical inoculation. Although CMV is readily transmitted by mechanical inoculation from plant to plant in many species, its transmission to gladiolus is difficult. On rare occasions, we were able to achieve mechanical inoculation, but the infection was unpredictable and the rate was low. This is probably due to the nature and number of infectable sites on the leaf surface of gladiolus being different from other plants.

A method of inoculation that consistently gives reliable and repeatable results is very important in many aspects of experimental plant virology. In gladiolus, inoculation of CMV involves the use of aphid vectors. In transmission studies, aphids are starved before given an acquisition access feeding followed by inoculation feeding. Not all isolates of CMV are aphid transmissible. Only the vectorial cultures can be transmitted by aphids. Loss of transmissibility occurs in vectorial strains after a few mechanical transfers, a common phenomenon among vector-borne plant viruses. Once a vectorial strain becomes ex-vectorial, the virus is no longer inoculative in gladiolus preventing further use of the culture in ongoing studies.

The gene gun inoculation method resulted in predictable (100%) infection rates and, when performed under sterile conditions, acceptable survival rates. Drying the cormels did not increase the rate of infection. Cartridges can be stored for many months without loss of CMV viability. The method proved successful for the inoculation of three isolates of CMV. The amount of virus used to prepare the cartridges can be adjusted to suit the needs of the study being performed. As little as 2 μg of virus can be used in cartridge preparation, yielding enough cartridges to inoculate 30 or 40 glad cormels and ensure 100% infection. This method of biolistic inoculation of CMV into gladiolus will be useful in studies of CMV transmission and disease resistance.

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